

1007 Rec'd PCT/PTO 14 FEB 2002

SUBSTITUTE FORM PTO-1390

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

ATTORNEY'S DOCKET NUMBER  
10848-018001

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)  
**10/049574**

INTERNATIONAL APPLICATION NO  
PCT/DE00/02748

INTERNATIONAL FILING DATE  
11 August 2000

PRIORITY DATE CLAIMED  
14 August 1999

TITLE OF INVENTION  
METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING STATUS

APPLICANT(S) FOR DO/EO/US  
Wolf Bertling

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☒ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))

Items 11 to 16 below concern other documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

☒ PCT International Search Report (7 pages) with seven references attached

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**CERTIFICATE OF MAILING BY EXPRESS MAIL**

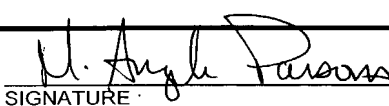
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I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, DC 20231

**14 February 2002**  
Date of Deposit

Signature

**VINCE DEFANTE**  
Typed Name of  
Person Signing

U.S. APPLICATION NO. (IF KNOWN) <b>10/049574</b>		INTERNATIONAL APPLICATION NO. PCT/DE00/02748		ATTORNEY'S DOCKET NUMBER 10848-018001	
17. <input checked="" type="checkbox"/> The following fees are submitted:  <b>Basic National Fee ( 37 CFR 1.492(a)(1)- (5) ):</b>  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1040</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>	
Surcharge of <b>\$130</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	16 - 20 =		x <b>\$18</b>	\$0.00	
Independent Claims	1 - 3 =		x <b>\$84</b>	\$0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ <b>\$280</b>	\$0.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$0.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2				\$445.00	
<b>SUBTOTAL =</b>				\$445.00	
Processing fee of <b>\$130</b> for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$0.00	
<b>TOTAL NATIONAL FEE =</b>				\$445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +				\$0.00	
<b>TOTAL FEES ENCLOSED =</b>				\$445.00	
				<b>Amount to be refunded:</b>	\$
				<b>Charged:</b>	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$445.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 06-1050 in the amount of \$0.00 to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:					
Mark S. Ellinger, Ph.D. FISH & RICHARDSON P.C., P.A. 60 South Sixth Street Suite 3300 Minneapolis, MN 55402 (612) 335-5070 phone (612) 288-9696 facsimile			SIGNATURE   NAME M. Angela Parsons, Ph.D.  REGISTRATION NUMBER 44,282		

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Attorney's Docket No.: 10848-018001 / 412023GA-go

JC12 Rec'd PCT/PTO 14 FEB 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#3/a

Applicant : Wolf Bertling  
Serial No. : To Be Assigned  
Filed : 14 February 2002  
Title : METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING  
STATUS

Art Unit : Unknown  
Examiner : Unknown

Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Specification:

Please add the following paragraph to the application after the title:

**--CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a National Stage application under 35 U.S.C. §371 and claims benefit under 35 U.S.C. §119(a) of International Application No. PCT/DE00/02748 having an International Filing Date of August 11, 2000, which claims benefit of DE 199 37 654.9 filed on August 14, 1999 and DE 199 41 447.5 filed on August 31, 1999.--

Please delete the paragraph on page 3, lines 29-31.

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Date of Deposit February 14, 2002

Signature 

Vince Defante  
Typed or Printed Name of Person Signing Certificate

Applicant : Wolf Bertling  
Serial No. :  
Filed :  
Page : 2

Attorney's Docket No.: 10848-018001 / 412023GA-go

In the Claims:

Please amend the claims as follows. A full set of pending claims are shown herein for convenience.

1. (Amended) A method for indirectly determining blood clotting status having the following steps:

a) providing a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase,

b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),

c) forming a first ratio (R1) from the first (C1) and second concentration (C2) or forming a second ratio (R2) from the third (C3) and first concentration (C1) or forming a third ratio (R3) from the third (C3) and second concentration (C2),

where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

$$C3 - C2 = C1$$

and

d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

2. The method as claimed in claim 1, where in step b) additionally at least a first competitor (K1) is used to measure the first concentration (C1), a second competitor (K2) is used to measure the second concentration (C2) or a third competitor (K3) is used to measure the third concentration (C3).

Applicant : Wolf Bertling  
 Serial No. :  
 Filed :  
 Page : 3

Attorney's Docket No.: 10848-018001 / 412023GA-go

3. (Amended) The method as claimed in claim 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a labeling substance.

4. (Amended) The method as claimed in claim 2, where in place of measuring the at least two concentrations as in step b), a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

5. The method as claimed in claim 4, where the combined signal is a combined color generated in particular by fluorescent dyes, a fluorescent signal elicited by the Förster effect or a reduction caused by the quencher in a fluorescent signal.

6. (Amended) The method as claimed in claim 1, where the body fluid is plasma, blood, saliva, urine or the like.

7. (Amended) The method as claimed in claim 1, where the measurement of the first (C1), second (C2) and/or third concentration (C3) or of the combined signal takes place by an immunological method.

8. (Amended) The method as claimed in claim 7, where in the immunological method, at least one of the antibodies (A1, A2, A3) is immobilized on a support.

9. (Amended) The method as claimed in claim 1, where the first (C1), second (C2) and/or third concentration (C3) and/or the combined signal is measured by means of a color reaction or fluorescence detection.

10. (Amended) The method as claimed in claim 1, where the protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

11. (Amended) A kit for carrying out the method as claimed in claim 1, having a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein and having a second antibody (A2) for immunological determination of a second concentration (C2) of the decarboxylated form of the protein, characterized in that the first (A1) and second antibodies (A2) is in each case conjugated to a labeling substance, where the labeling substances are selected so that they are able together to generate a combined signal.

Applicant : Wolf Bertling  
 Serial No. :  
 Filed :  
 Page : 4

Attorney's Docket No.: 10848-018001 / 412023GA-go

12. A kit as claimed in claim 11, where the labeling substance is an enzyme, a fluorescent dye or a quencher.

13. (Amended) A kit as claimed in claim 11, where the combined signal is a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal.

14. (Amended) A kit as claimed in claim 11, where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

Please cancel claims 15-23 without prejudice to continued prosecution.

Please add the following new claims:

24. The method of claim 3, wherein the labeling substance is selected from the group consisting of an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin, and avidin.

25. The method of claim 8, wherein the support is selected from the group consisting of a plastic, a magnetic particle, a latex particle, a gold particle, a test strip, and a membrane.

In the Abstract:

Please add the attached Abstract to the application after the claims.

Applicant : Wolf Bertling  
Serial No. :  
Filed :  
Page : 5

Attorney's Docket No.: 10848-018001 / 412023GA-go

### REMARKS

Applicant respectfully requests entry of the amendments and remarks submitted herein. Claims 1, 3-4, 6-11, and 13-14 have been amended, claims 15-23 have been canceled, and new claims 24-25 have been added. Support for the amendments and for new claims 24-25 can be found in the originally filed claims and throughout the specification. Therefore, claims 1-14 and 24-25 are currently pending. Attached is a marked-up version of the changes being made by the current amendments. Reconsideration of the pending application is respectfully requested.

In addition, Applicants amended the specification to include a paragraph describing related applications and to claim the benefit of priority to such applications. Applicants also have amended the specification to remove the paragraph on page 3 that refers to claim numbers, and to add an Abstract. The attached Abstract is the English language Abstract that was published with the PCT application. Therefore, Applicants submit that there is no new matter introduced by these amendments.

### CONCLUSION

Applicant asks that claims 1-14 and 24-25 be examined. The enclosed filing fee takes into account the new claims added by this Preliminary Amendment. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: February 14, 2002

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M. Angela Parsons, Ph.D.  
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Applicant : Wolf Bertling  
 Serial No. :  
 Filed :  
 Page : 6

Attorney's Docket No.: 10848-018001 / 412023GA-go

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

The paragraph on page 3, lines 29-31 has been deleted.

In the Claims:

Claims 1, 3-4, 6-11, and 13-14 have been amended as follows:

1. (Amended) A method for indirectly determining [the] blood clotting status having the following steps:

- a) providing [provision of] a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase,
- b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),
- c) forming a first ratio (R1) from the first (C1) and second concentration (C2) or forming a second ratio (R2) from the third (C3) and first concentration (C1) or forming a third ratio (R3) from the third (C3) and second concentration (C2),

where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

$$C3 - C2 = C1$$

and

- d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

3. (Amended) The method as claimed in claim [1 or] 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a



Applicant : Wolf Bertling  
 Serial No. :  
 Filed :  
 Page : 7

Attorney's Docket No.: 10848-018001 / 412023GA-go

labeling substance[, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, a biotin, streptavidin or avidin].

4. (Amended) The method as claimed in claim 2 [any of the preceding claims], where in place of measuring the at least two concentrations as in step b), a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

6. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the body fluid is plasma, blood, saliva, urine or the like.

7. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the measurement of the first (C1), second (C2) and/or third concentration (C3) or of the combined signal takes place by an immunological method.

8. (Amended) The method as claimed in claim 7, where in the immunological method, at least one of the antibodies (A1, A2, A3) is immobilized on a support[, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane].

9. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the first (C1), second (C2) and/or third concentration (C3) and/or the combined signal is measured by means of a color reaction or fluorescence detection.

10. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

11. (Amended) A kit for carrying out the method as claimed in claim 1 [any of the preceding claims], having a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein and having a second antibody (A2) for immunological determination of a second concentration (C2) of the decarboxylated form of the protein, characterized in that the first (A1) and second antibodies (A2) is in each case conjugated to a labeling substance, where the labeling substances are selected so that they are able together to generate a combined signal.

Applicant : Wolf Bertling  
Serial No. :  
Filed :  
Page : 8

Attorney's Docket No.: 10848-018001 / 412023GA-go

13. (Amended) A kit as claimed in claim 11 [or 12], where the combined signal is a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal.

14. (Amended) A kit as claimed in [any of claims] claim 11 [to 13], where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

Claims 15-23 have been canceled.

New claims 24-25 have been added.

In the Abstract:

The Abstract on the attached page has been added to the application.

**ABSTRACT OF THE DISCLOSURE**

The invention relates to a method for indirectly determining the blood-clotting status. The inventive method comprises the following steps: a) collecting body fluids which contain a protein that can be modified by a vitamin K-dependent  $\gamma$ -carboxylase, b) determining at least two concentrations selected from a group consisting of a first concentration C1 of carboxylated protein, a second concentration C2 of decarboxylated protein and an entire concentration C3 of carboxylated and decarboxylated protein, whereby the first concentration C1 is determined using a first antibody A1, the second concentration using a second antibody A2 and the third concentration C3 using a third antibody A3, c) generating a first quotient Q1 from the first C1 and second C2 concentration or generating a second quotient Q2 from the third C3 and first C1 concentration or generating a third quotient Q3 from the third C3 and second concentration C2, whereby a concentration C1, C2, C3 which has not been determined in step b) and which is required for generating the first Q1, the second Q2, or the third quotient Q3 is calculated according to the following relation:  $C3 - C2 = C1$  and d) the first, second or third quotient Q1, Q2, Q3 are correlated with the blood-clotting status.

**Method for indirectly determining the blood clotting  
status**

The invention relates to a method for indirectly  
5 determining the blood clotting status.

The blood clotting status can be determined indirectly  
by measuring the prothrombin concentration in human  
body fluids. Prothrombin is a protein which occurs  
10 chiefly in human blood plasma. This protein can be  
modified by a vitamin K-dependent  $\gamma$ -carboxylase.  
Prothrombin is partly responsible for blood clotting.  
It converts fibrinogen into fibrin.

15 The fibrinogen conversion induced by prothrombin takes  
place only when prothrombin is present in natural  
carboxylated form. The carboxylation is effected in the  
liver by a carboxylase with binding of the cofactor  
vitamin K. The carboxylase activity depends on the  
20 vitamin K concentration. If the carboxylase activity is  
reduced, an abnormal, noncarboxylated form of  
prothrombin which has no clotting activity is produced.

In healthy people, prothrombin is present in the  
25 natural, i.e. carboxylated, form. Vitamin K acts as  
cofactor in the carboxylation. In people who are ill,  
especially in people with liver damage, or on addition  
of anticoagulants, prothrombin also occurs in the  
abnormal form.

30 Carboxylated prothrombin brings about clotting only if  
 $\text{Ca}^{2+}$  ions are bound beforehand. Only then is the  
carboxylated prothrombin able to bind to the membranes  
of blood platelets and bring about clotting. Only the  
35 carboxylated form of prothrombin is able to bind  
calcium. The blood clotting status can thus be inferred  
from the content of carboxylated prothrombin.

- 2 -

US 4,769,320 discloses a method in which the content of carboxylated prothrombin is measured by use of antibodies in an immunoassay. The antibodies are specific for carboxylated prothrombin in the presence of  
5 calcium. They do not bind to decarboxylated prothrombin. A kit for determining the level of carboxylated prothrombin in a plasma sample is described and contains such an antibody.

10 US 5,252,712 discloses a monoclonal antibody which is specific for noncarboxylated prothrombin. It is possible by use of this antibody in an immunoassay to measure the concentration of uncarboxylated  
15 prothrombin. This also makes it possible to gain information about the blood clotting status.

US 4,780,410 discloses a sandwich immunoassay method for quantifying decarboxylated prothrombin. An immobilized monoclonal antibody directed against  
20 decarboxylated prothrombin is used in the method. Decarboxylated prothrombin binding thereto is detected by a second antibody directed against prothrombin. A kit for carrying out the method is also described.

25 Kornberg A. et al., Circulation 88 (1993), pages 454 - 460, disclose determination of the concentration of carboxylated prothrombin in a sample by use of a competitor. In this case, peroxidase-labeled prothrombin competes as competitor with the prothrombin  
30 in the sample for binding to an immobilized anti-prothrombin antibody. The peroxidase-labeled prothrombin bound to the anti-prothrombin antibody can be detected by an enzyme reaction. The size of the signal produced thereby is inversely proportional to  
35 the prothrombin concentration in the sample.

JP 05 284 994 A discloses three monoclonal antibodies. A first one binds specifically to human decarboxylated prothrombin, a second one binds specifically to human



- 4 -

- b) measuring at least two concentrations selected from a group consisting of a first concentration of carboxylated protein, a second concentration of decarboxylated protein and a total concentration of carboxylated and decarboxylated protein, where the first concentration is measured using a first antibody, the second concentration is measured using a second antibody and the third concentration is measured using a third antibody,
- c) forming a first ratio from the first and second concentration
- or
- forming a second ratio from the third and first concentration
- or
- forming a third ratio from the third and second concentration,
- where a concentration which is necessary for forming the first, second or third ratio and is not measured in step b) is calculated in accordance with the following relation:
- $$C3 - C2 = C1$$
- and
- d) correlating the first, second or third ratio with the blood clotting status.

A protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase means a protein which, depending on the blood clotting status, may be present in proportions





- 6 -

used as enzyme. The labeling of the antibody with a gold particle permits the bound antibody to be detected by a plasmon resonance method.

5 In place of measuring the at least two concentrations as in step b) it is also possible for a combined signal correlating therewith to be generated and measured by using two antibodies selected from a group consisting of the first, the second and the third antibody and, 10 where appropriate, at least one of the competitors, and be directly correlated with the blood clotting status. The combined signal is produced by different individual signals acting together. It corresponds to the first, second or third ratio. The formation of these ratios as 15 in step c) is dispensed with. This makes it possible to carry out the method more quickly and simply. The combined signal may be a combined color, generated in particular by fluorescent dyes, a fluorescent signal elicited by the Förster effect or a reduction caused by 20 the quencher in a fluorescent signal. The combined color may also be generated by two enzymes which each catalyze a specific color reaction. The Förster effect involves a radiationless energy transfer from an excited first fluorophore to a directly adjacent second 25 fluorophore. The first fluorophore thus makes the transition to the ground state, while the second fluorophore is excited and fluoresces. In the method of the invention it is possible, for example, for the first and second antibodies to be conjugated with 30 fluorophores which make the first effect possible. The binding of the first antibodies directly adjacent to the second antibodies can be established by means of a fluorescent signal elicited by the Förster effect. When there is a radiationless energy transfer from 35 fluorophore to quencher the fluorescence is quenched. The total measurable fluorescent signal is thus reduced.

- 7 -

The body fluid may expediently be plasma, blood, saliva, urine or the like. All body fluids in which the modifiable protein is present in a content which makes measurement possible are suitable in principle.

5

According to one developmental feature of the invention, the measurement of the first, second and/or third concentration or of the combined signal takes place by an immunological method. The immunological method in  
10 this case may involve at least one of the antibodies being immobilized on a support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane. The plastic may be in the form of a test tube, a test strip, a plastic  
15 particle or a well of a microtiter plate.

The first, second and/or third concentration and/or the combined signal can be measured by means of a color reaction or fluorescence detection. This makes possible  
20 particularly rapid and simple measurement of the blood clotting status.

The protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase is preferably one of the  
25 clotting factors which are referred to in the decarboxylated form as "proteins induced by vitamin K antagonism or absence" (PIVKA factors): prothrombin, factor VII, factor IX or factor X, or is nephrocalcin or osteocalcin. It is also possible to use for  
30 determining the blood clotting status other proteins which are carboxylated by a vitamin K-dependent carboxylase and can likewise be influenced by anticoagulants which can be administered orally. Nephrocalcin is, for example, detectable in urine. It  
35 is unnecessary to take any blood on use of this protein. This means a considerable alleviation for patients whose blood clotting status must be monitored continuously.

- 8 -

Also provided is a kit for carrying out the method of the invention, comprising at least two antibodies selected from a group consisting of a first antibody for immunological determination of a first concentration of the carboxylated form of the protein, a second antibody for immunological determination of a second concentration of the decarboxylated form of the protein and a third antibody for immunological determination of a total concentration of carboxylated and decarboxylated protein.

The first, second and third antibody may be antibodies known in the prior art. Such antibodies are disclosed, for example, in US 5,252,712 and US 4,769,320, the contents of which are incorporated herein by reference.

The kit may additionally comprise at least a first competitor for measuring the first concentration, a second competitor for measuring the second concentration or a third competitor for measuring the third concentration. At least one of the antibodies or competitors present in the kit may be conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin.

The first and the second antibody are preferably immobilized on a support. The support may be a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane. If the support is a test strip, the first and the second antibody may each be absorbed on a separate field on the test strip. The kit preferably comprises a third antibody conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin. This makes it possible to measure the respective content particularly simply, for example by means of a color reaction on the test strip.



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in PBS/0.1% BSA) are added. The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20.

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50  $\mu$ l/cavity of strepavidin-peroxidase conjugate (Roche Diagnostics, 1:1000 in conjugate buffer) are added. The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20.

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To carry out the development reaction, 50  $\mu$ l/cavity of ABTS solution (Roche Diagnostics, 1 mg/ml) are added. The microtiter plate is incubated at room temperature for half an hour to one hour. The absorptions (OD values) are measured in an ELISA reader.

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It is self-evident that the method can be considerably shortened if the anti-prothrombin antibody used for detecting bound prothrombin already has a labeling substance such as peroxidase or another enzyme. A further shortening of the method can be achieved by using a directly detectable labeling substance such as a fluorophore. No development reaction is necessary on use of such a labeling substance.

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b) Evaluation:

The following are determined:

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aa) the OD values of the complete prothrombin (cavities are coated with monoclonal anti-complete prothrombin antibodies),

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bb) the OD values of the decarboxylated prothrombin (cavities are coated with monoclonal anti-decarboxy-prothrombin antibodies) and

cc) the OD values of the carboxylated prothrombin  
(difference between the OD values of the complete

- 12 -

prothrombin and the OD values of the decarboxylated prothrombin).

5 Then calibration plots are constructed from the measured OD values (see figs. 1 - 3: points A, B, C and D) of the calibration plasmas, and the INR of the patient's plasmas calculated.

10 It is self-evident that the clotting status can also be measured by measuring the corresponding concentrations of proteins other than prothrombin which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase. Examples of such proteins are factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

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Example 2:

The cavities of a microtiter plate (Maxisorb, NUNC) are coated with in each case 50  $\mu$ l of an antibody directed against carboxylated and decarboxylated prothrombin or  
20 of an antibody directed only against decarboxylated prothrombin (10  $\mu$ g/ml in carbonate buffer) at 4°C overnight. The cavities are washed three times with PBS. Nonspecific binding sites are saturated with 50  $\mu$ l of 1% BSA in PBS per cavity at room temperature for one  
25 hour. The cavities are then washed three times with PBS/0.05% Tween 20.

Peroxidase-labeled decarboxylated prothrombin is pulled together with the following samples, each diluted 1:50  
30 in PBS/0.1% BSA, in a final concentration of 30  $\mu$ g/ml into the antibody-coated cavities of the microtiter plate (50  $\mu$ l/cavity):

calibration plasmas,  
35 normal plasma,  
patient's plasma and  
prothrombin-deficient plasma (negative control).





- 1 -

## New claims

1. A method for indirectly determining the blood clotting status having the following steps:

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a) provision of a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent  $\gamma$ -carboxylase,

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b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),

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c) forming a first ratio (R1) from the first and second concentration (C2)

or

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forming a second ratio (R2) from the third (C3) and first concentration (C1)

or

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forming a third ratio (R3) from the third (C3) and second concentration (C2),

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where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

- 2 -

$$C3 - C2 = C1$$

and

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d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

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2. The method as claimed in claim 1, where in step b) additionally at least a first competitor (K1) is used to measure the first concentration (C1), a second competitor (K2) is used to measure the second concentration (C2) or a third competitor (K3) is used to measure the third concentration (C3).

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3. The method as claimed in claim 1 or 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, a biotin, streptavidin or avidin.

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4. The method as claimed in any of the preceding claims, where in place of measuring the at least two concentrations as in step b) a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

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5. The method as claimed in claim 4, where the combined signal is a combined color generated in particular by fluorescent dyes, a fluorescent





- 5 -

16. The use as claimed in claim 15, where additionally at least a first competitor (K1) is present for measuring the first concentration (C1), a second competitor (K2) is present for measuring the second concentration (C2) or a third competitor (K3) is present for measuring the third concentration (C3).
17. The use as claimed in claim 15 or 16, where at least one of the antibodies (A1, A2, A3) or competitors (K1, K2, K3) present is conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin.
18. The use as claimed in any of claims 15 to 17, where the first (A1) and the second antibody (A2) are immobilized on a support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane.
19. The use as claimed in claim 18, where the support is a test strip, and the first (A1) and the second antibody (A2) are each absorbed on a separate field of the test strip.
20. The use as claimed in claim 18 or 19, where a third antibody (A3) conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin is present.
21. The use as claimed in any of claims 15 to 20, where the third antibody (A3) is immobilized on the or another support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane.

- 6 -

22. The use as claimed in claim 21, where the support is the or another test strip, and the third antibody (A3) is absorbed on a field of the test strip.

5

23. The use as claimed in any of claims 15 to 22, where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

(12) NACH DEM VERFAHREN ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES  
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

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**Veröffentlicht:**

— Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING STATUS

(54) Bezeichnung: VERFAHREN ZUR INDIREKTEN BESTIMMUNG DES BLUTGERINNUNGSSTATUS

(57) Abstract: The invention relates to a method for indirectly determining the blood-clotting status. The inventive method comprises the following steps: a) collecting body fluids which contain a protein that can be modified by a vitamin K-dependent  $\gamma$ -carboxylase, b) determining at least two concentrations selected from a group consisting of a first concentration C1 of carboxylated protein, a second concentration C2 of decarboxylated protein and an entire concentration C3 of carboxylated and decarboxylated protein, whereby the first concentration C1 is determined using a first antibody A1, the second concentration using a second antibody A2 and the third concentration C3 using a third antibody A3, c) generating a first quotient Q1 from the first C1 and second concentration C2 or generating a second quotient Q2 from the third C3 and first concentration C1 or generating a third quotient Q3 from the third C3 and second concentration C2, whereby a concentration C1, C2, C3 which has not been determined in step b) and which is required for generating the first Q1, the second Q2 or the third quotient Q3 is calculated according to the following relation:  $C3 \cdot C2 = C1$  and d) the first, second or third quotient Q1, Q2, Q3 are correlated with the blood-clotting status.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur indirekten Bestimmung des Blutgerinnungsstatus mit folgenden Schritten: a) Entnahme von Körperflüssigkeit, die ein durch eine Vitamin-K abhängige  $\gamma$ -Carboxylase modifizierbares Protein enthält; b) Ermittlung von mindestens zwei Konzentrationen ausgewählt aus einer Gruppe bestehend aus einer ersten Konzentration (C1) an carboxyliertem Protein, einer zweiten Konzentration (C2) an decarboxyliertem Protein und einer Gesamtkonzentration (C3) an carboxyliertem und decarboxyliertem Protein, wobei die erste Konzentration (C1) unter Verwendung eines ersten Antikörpers (A1), die zweite Konzentration unter Verwendung eines zweiten Antikörpers (A2) und die dritte Konzentration (C3) unter Verwendung eines dritten Antikörpers (A3) ermittelt wird; c) Bildung eines ersten Quotienten (Q1) aus erster (C1) und zweiter Konzentration (C2), oder Bildung eines zweiten Quotienten (Q2) aus dritter (C3) und erster Konzentration (C1), oder Bildung eines dritten Quotienten (Q3) aus dritter (C3) und zweiter Konzentration (C2), wobei eine zur Bildung des ersten (Q1), zweiten (Q2) oder dritten Quotienten (Q3) erforderliche und bei Schritt lit. (b) nicht ermittelte Konzentration (C1, C2, C3) gemäß folgender Beziehung:  $C3 \cdot C2 = C1$  errechnet wird; und d) Korrelation des ersten, zweiten oder dritten Quotienten (Q1, Q2, Q3) mit dem Blutgerinnungsstatus.

WO 01/13123 A2

WO 01/13123

PCT/DE00/02748

1 / 3

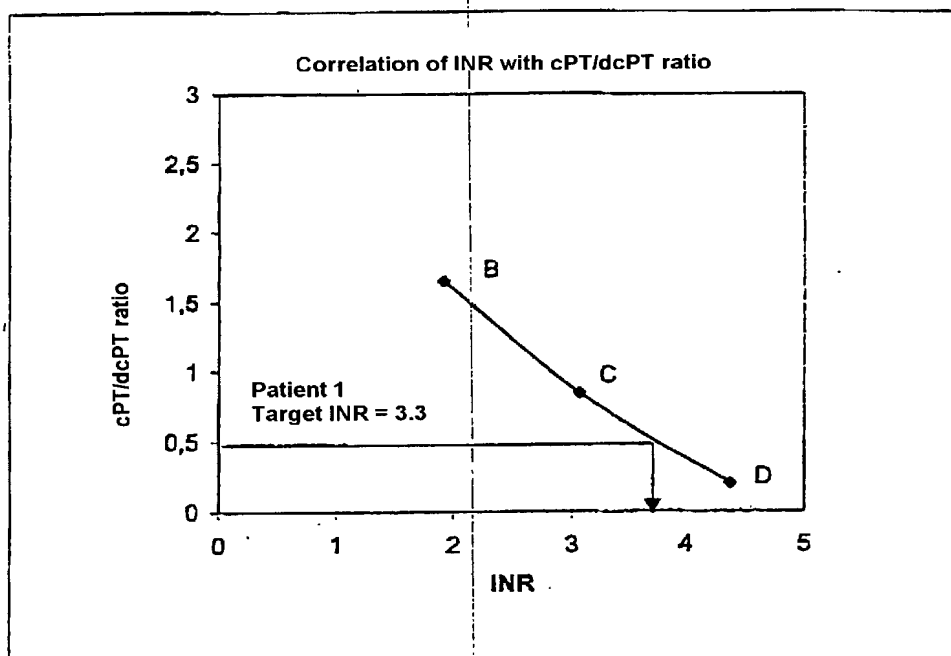


Fig. 1



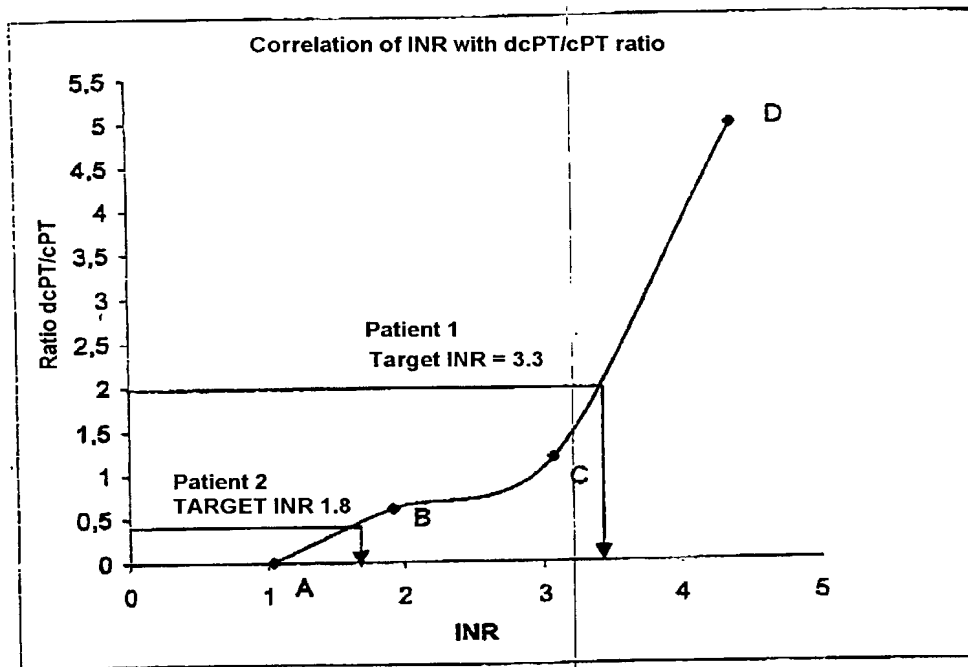


Fig. 2

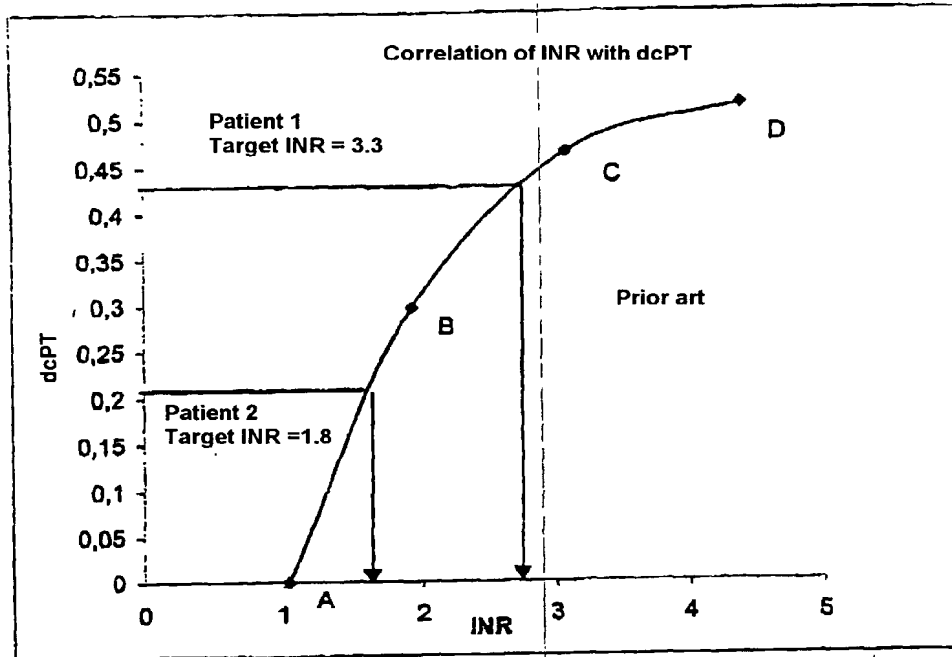


Fig. 3

1984-1985 1986-1987 1988-1989 1990-1991 1992-1993 1994-1995 1996-1997 1998-1999 2000-2001 2002-2003 2004-2005 2006-2007 2008-2009 2010-2011 2012-2013 2014-2015 2016-2017 2018-2019 2020-2021 2022-2023 2024-2025 2026-2027 2028-2029 2030-2031 2032-2033 2034-2035 2036-2037 2038-2039 2040-2041 2042-2043 2044-2045 2046-2047 2048-2049 2050-2051 2052-2053 2054-2055 2056-2057 2058-2059 2060-2061 2062-2063 2064-2065 2066-2067 2068-2069 2070-2071 2072-2073 2074-2075 2076-2077 2078-2079 2080-2081 2082-2083 2084-2085 2086-2087 2088-2089 2090-2091 2092-2093 2094-2095 2096-2097 2098-2099 2100-2101 2102-2103 2104-2105 2106-2107 2108-2109 2110-2111 2112-2113 2114-2115 2116-2117 2118-2119 2120-2121 2122-2123 2124-2125 2126-2127 2128-2129 2130-2131 2132-2133 2134-2135 2136-2137 2138-2139 2140-2141 2142-2143 2144-2145 2146-2147 2148-2149 2150-2151 2152-2153 2154-2155 2156-2157 2158-2159 2160-2161 2162-2163 2164-2165 2166-2167 2168-2169 2170-2171 2172-2173 2174-2175 2176-2177 2178-2179 2180-2181 2182-2183 2184-2185 2186-2187 2188-2189 2190-2191 2192-2193 2194-2195 2196-2197 2198-2199 2200-2201 2202-2203 2204-2205 2206-2207 2208-2209 2210-2211 2212-2213 2214-2215 2216-2217 2218-2219 2220-2221 2222-2223 2224-2225 2226-2227 2228-2229 2230-2231 2232-2233 2234-2235 2236-2237 2238-2239 2240-2241 2242-2243 2244-2245 2246-2247 2248-2249 2250-2251 2252-2253 2254-2255 2256-2257 2258-2259 2260-2261 2262-2263 2264-2265 2266-2267 2268-2269 2270-2271 2272-2273 2274-2275 2276-2277 2278-2279 2280-2281 2282-2283 2284-2285 2286-2287 2288-2289 2290-2291 2292-2293 2294-2295 2296-2297 2298-2299 2300-2301 2302-2303 2304-2305 2306-2307 2308-2309 2310-2311 2312-2313 2314-2315 2316-2317 2318-2319 2320-2321 2322-2323 2324-2325 2326-2327 2328-2329 2330-2331 2332-2333 2334-2335 2336-2337 2338-2339 2340-2341 2342-2343 2344-2345 2346-2347 2348-2349 2350-2351 2352-2353 2354-2355 2356-2357 2358-2359 2360-2361 2362-2363 2364-2365 2366-2367 2368-2369 2370-2371 2372-2373 2374-2375 2376-2377 2378-2379 2380-2381 2382-2383 2384-2385 2386-2387 2388-2389 2390-2391 2392-2393 2394-2395 2396-2397 2398-2399 2400-2401 2402-2403 2404-2405 2406-2407 2408-2409 2410-2411 2412-2413 2414-2415 2416-2417 2418-2419 2420-2421 2422-2423 2424-2425 2426-2427 2428-2429 2430-2431 2432-2433 2434-2435 2436-2437 2438-2439 2440-2441 2442-2443 2444-2445 2446-2447 2448-2449 2450-2451 2452-2453 2454-2455 2456-2457 2458-2459 2460-2461 2462-2463 2464-2465 2466-2467 2468-2469 2470-2471 2472-2473 2474-2475 2476-2477 2478-2479 2480-2481 2482-2483 2484-2485 2486-2487 2488-2489 2490-2491 2492-2493 2494-2495 2496-2497 2498-2499 2500-2501 2502-2503 2504-2505 2506-2507 2508-2509 2510-2511 2512-2513 2514-2515 2516-2517 2518-2519 2520-2521 2522-2523 2524-2525 2526-2527 2528-2529 2530-2531 2532-2533 2534-2535 2536-2537 2538-2539 2540-2541 2542-2543 2544-2545 2546-2547 2548-2549 2550-2551 2552-2553 2554-2555 2556-2557 2558-2559 2560-2561 2562-2563 2564-2565 2566-2567 2568-2569 2570-2571 2572-2573 2574-2575 2576-2577 2578-2579 2580-2581 2582-2583 2584-2585 2586-2587 2588-2589 2590-2591 2592-2593 2594-2595 2596-2597 2598-2599 2600-2601 2602-2603 2604-2605 2606-2607 2608-2609 2610-2611 2612-2613 2614-2615 2616-2617 2618-2619 2620-2621 2622-2623 2624-2625 2626-2627 2628-2629 2630-2631 2632-2633 2634-2635 2636-2637 2638-2639 2640-2641 2642-2643 2644-2645 2646-2647 2648-2649 2650-2651 2652-2653 2654-2655 2656-2657 2658-2659 2660-2661 2662-2663 2664-2665 2666-2667 2668-2669 2670-2671 2672-2673 2674-2675 2676-2677 2678-2679 2680-2681 2682-2683 2684-2685 2686-2687 2688-2689 2690-2691 2692-2693 2694-2695 2696-2697 2698-2699 2700-2701 2702-2703 2704-2705 2706-2707 2708-2709 2710-2711 2712-2713 2714-2715 2716-2717 2718-2719 2720-2721 2722-2723 2724-2725 2726-2727 2728-2729 2730-2731 2732-2733 2734-2735 2736-2737 2738-2739 2740-2741 2742-2743 2744-2745 2746-2747 2748-2749 2750-2751 2752-2753 2754-2755 2756-2757 2758-2759 2760-2761 2762-2763 2764-2765 2766-2767 2768-2769 2770-2771 2772-2773 2774-2775 2776-2777 2778-2779 2780-2781 2782-2783 2784-2785 2786-2787 2788-2789 2790-2791 2792-2793 2794-2795 2796-2797 2798-2799 2800-2801 2802

DATE: 11/11/2011

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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[REDACTED]

COMBINED DECLARATION AND POWER OF ATTORNEY

nov 10/1/02

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled *Method For Indirectly Determining the Blood-Clotting Status*, the specification of which:

- ☐ is attached hereto.  
☒ was filed on February 14, 2002 as Application Serial No. 10/049,574.  
☐ was described and claimed in PCT International Application No. \_\_\_\_\_ filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
WIPO	PCT/DE00/02748	August 11, 2000	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Germany	199 41 447.5	August 31, 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Germany	199 37 654. 9	August 14, 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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